



National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material[®] 870

Column Performance Test Mixture for Liquid Chromatography¹

SRM 870 is a mixture of five organic compounds in methanol intended for use in characterizing general aspects of liquid chromatographic (LC) column performance, including efficiency, void volume, methylene selectivity, retentiveness, and activity toward chelators and organic bases. Other possible uses include (1) column classification to aid column selection during method development, (2) as a control material for monitoring LC column performance over time, and (3) in quality control for column manufacturing. SRM 870 consists of a mixture of the following five organic compounds in methanol: uracil, toluene, ethylbenzene, quinizarin, and amitriptyline (see Figure 1 for structures). The concentrations and relative detection responses of the components are listed in Table 1. A unit of SRM 870 consists of 5 ampoules each containing 1.1 mL of the mixture. SRMs are also available for the characterization of other chromatographic properties including shape selectivity (i.e., SRM 869a *Column Selectivity Test Mixtures for Liquid Chromatography*) [2] and chiral selectivity (i.e., SRM 877 *Chiral Selectivity Test Mixture for Liquid Chromatography*) [3].

Expiration of Certification: SRM 870 is valid for its intended purpose until **30 September 2010**, provided the SRM is handled and stored in accordance with the instructions given in this certificate. The certification is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Return of the attached registration card will facilitate notification.

NOTICE AND WARNINGS TO USERS

Toxicity: This test mixture contains small amounts of organic compounds known to be toxic. Care should be exercised during handling and use (see *Instructions for Use*). Use proper methods for disposal of waste.

The coordination of the technical measurements leading to certification were performed under the direction of L.C. Sander and S.A. Wise of the NIST Analytical Chemistry Division.

Preparation and analytical determinations were carried out by L.C. Sander of the NIST Analytical Chemistry Division.

The support aspects involved in the preparation, certification, and issuance of this SRM were coordinated through the NIST Standard Reference Materials Group by B.S. MacDonald.

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Certificate Issue Date: 19 December 2002
See Certificate Revision History on Page 4

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¹Certain commercial equipment, instruments, or materials are identified in this certificate in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

INSTRUCTIONS FOR USE

Storage: Sealed ampoules, as received, should be stored in the dark at temperatures between 10 °C to 30 °C.

Chromatographic Conditions¹: This test mixture is intended primarily for the characterization of C₁₈ columns used in reversed-phase liquid chromatography. To compare columns on the same basis, the user should evaluate column performance by separating the mixture isocratically under the following conditions: mobile phase, 80 % methanol and 20 % buffer (v/v), flow rate 1 mL/min, column temperature 23 °C, injection volume 1 µL to 2 µL. The recommended buffer composition is 20 mmol/L potassium phosphate adjusted to pH 7.0 (final phosphate concentration in the mixed methanol/buffer mobile phase is 4 mmol/L). This buffer can be prepared by mixing 20 mmol/L monobasic potassium phosphate (KH₂PO₄) and 20 mmol/L dibasic potassium phosphate (K₂HPO₄) solutions to obtain the desired pH 7.0 solution, as indicated by a pH meter. Because changes in absolute retention, selectivity, and peak shape can occur with changes in temperature and composition, these conditions should be used for all column evaluations.

INTERPRETATION OF RESULTS

Separations of the test mixture are illustrated in Figures 2A through 2F for several different C₁₈ columns. These chromatograms are representative examples of possible types of retention behavior. The most typical elution order is shown in Figure 2C. Uracil elutes near the void volume, followed by toluene and ethylbenzene. The elution order for quinizarin and amitriptyline varies with column properties. Quinizarin may elute before, after, or coelute with amitriptyline.

In most instances, peak identification can be made on the basis of elution order (uracil, toluene, ethylbenzene) and detector response (quinizarin, amitriptyline). Relative peak areas are dependent on the detection wavelength (see Table 1). Quinizarin has significant absorbance at 480 nm, and separations of SRM 870 carried out at this wavelength are selective for this single component. Conversely, quinizarin exhibits reduced absorbance at 210 nm, permitting measurement of amitriptyline in the presence of quinizarin. A comparison of separations carried out with detection at 210 nm, 254 nm, and 480 nm is provided in Figure 3. In the event of coelution of quinizarin and amitriptyline, data for each component can often be obtained by selective detection at 210 nm and 480 nm (see Table 1). At 210 nm, the area of quinizarin is approximately 2 % of the area of amitriptyline, making the interference to amitriptyline small.

The retention behavior of reversed-phase LC columns often differs in a variety of ways. The components in this test mixture were selected as indicators of several types of chromatographic properties. The determination of peak width (efficiency; theoretical plates), peak asymmetry (A_s), absolute retention (k'), and selectivity factor (α , i.e., relative retention k'_1/k'_2) for these components may provide useful measures of these properties. See Reference 1 for a discussion of the calculation of these parameters.

Uracil: This component is commonly used as an indicator of the void volume (unretained volume) in an LC column. The measurement of void volume is somewhat controversial; however, uracil provides an acceptable approximation of this property.

Toluene/Ethylbenzene: The retention of these compounds can be considered to result primarily from solvophobic interactions. The selectivity factor $\alpha_{E/T}$ is the k' ratio of ethylbenzene and toluene, and this value has been used to characterize differences among C₁₈ and C₈ columns. Absolute retention of a nonpolar component such as ethylbenzene provides a measure of column retentiveness (column strength). Toluene and/or ethylbenzene are also useful markers for calculation of column efficiency (theoretical plates, N).

Quinizarin: Quinizarin (1,4-dihydroxyanthraquinone) is a metal chelating reagent (see Figure 1). The retention behavior of this component is expected to be indicative of the presence or absence of metals in the chromatographic system. Columns demonstrate one of two types of retention behavior. Low activity toward chelating reagents is indicated by symmetric peak shape, and high activity toward chelating reagents is indicated by tailing, asymmetric peak shape. Quinizarin typically elutes after ethylbenzene and before amitriptyline. It is interesting to note that for columns known to contain certain embedded polar functional groups, quinizarin elutes last, with good peak symmetry. Peak asymmetry is not strongly correlated with retention for quinizarin.

¹The test conditions have been slightly altered from conditions previously described to expand the utility of the test.

Amitriptyline: Amitriptyline is a basic ($pK_a = 9.4$) pharmaceutical drug (antidepressant) commonly used by column manufacturers for column characterization. Elution of organic bases with severe peak tailing is often associated with high silanol activity; however, the elution of such compounds with symmetrical peak shape is considered indicative of column deactivation. Because peak tailing is the most objectionable property associated with silanol activity, A_s is an appropriate measure of this property. Peak asymmetry is not strongly correlated with retention for amitriptyline.

DISCUSSION

Selection of the components in SRM 870 was based on published testing protocols [4,5] and commercial column literature [6]. An effort was made to provide a simple, easy to evaluate test with a limited number of components. Component concentrations were adjusted to facilitate identification. This test is not intended for column classification as “good” or “bad”; however, columns that exhibit certain properties may be more suitable for a given application than others.

Test Conditions: The influence of chromatographic conditions on test results was examined for several different parameters. Relative changes in retention have been evaluated in reference 4 for pH, temperature, buffer concentration, and mobile phase composition. Because retention, efficiency, and peak shape are influenced by testing conditions, column evaluation should be carried out under standardized conditions to facilitate column comparisons. The largest changes in retention behavior occur with changes in the mobile phase composition. As specified in the *Instructions for Use* section of this certificate, the recommended composition of the mobile phase is 80 % methanol and 20 % buffer, where the buffer composition is 20 mmol/L potassium phosphate adjusted to pH 7.0 ± 0.1 . The retention of quinizarin and amitriptyline is strongly dependent on the pH of the potassium phosphate buffer solution (see Figure 4). The retention of quinizarin is reduced at high pH, whereas the retention of amitriptyline is reduced at low pH. At pH 7.0, both solutes exhibit significant retention. The ionic strength of the buffer is less significant. Only slight changes in retention, efficiency, and peak asymmetry are measurable with changes on the phosphate buffer concentration at pH 7.0. The presence of the buffer is essential, however. At levels below 1 mmol/L (buffer concentration before dilution with methanol), A_s and k' increase dramatically for amitriptyline. The absolute retention of the polar and nonpolar components increase with the percentage of buffer in the mobile phase (at pH 7.0, and constant ionic strength in the mixed solution). A composition of 80 % methanol and 20 % buffer was selected to provide appropriate retention for a broad range of column types.

Injection volume can also significantly influence test results (see Figure 5). Separation efficiency typically decreases with increased injection volume. Injection overload results in degraded peak shape and in some instances, reduced retention. An injection volume of 1 μ L to 2 μ L is recommended for 4.6 mm i.d. columns. Proportionately smaller injection volumes may be required for smaller i.d. columns.

Changes in column temperature influence the absolute retention of the components in SRM 870; however, relatively small effects are observed in the peak shape of quinizarin or amitriptyline. It is recommended that column temperature be controlled to $23\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.

Column Comparisons: Forty-one commercial C_{18} columns were utilized in the development of SRM 870. Columns were selected to represent a broad sampling of chromatographic retention properties, and included alkyl phases prepared with embedded polar functional groups. No two columns exhibit identical retention behavior; however, similarities do exist among several columns. Among columns utilized, values of k' for ethylbenzene ranged from 0.2 to 2.8. In contrast, only slight differences were observed for methylene selectivity ($\alpha_{E/T}$; range, 1.26 to 1.45). The retention of quinizarin ranged from $k' = 1$ to $k' = 23.6$. In two instances, no elution of this compound was detected. Peak asymmetry values ranged from $A_s = 1.1$ to $A_s = 5.7$ (peaks were not defined well enough in two instances to permit determination of A_s). Finally, the retention of amitriptyline ranged from $k' = 1.4$ to $k' = 72.9$ ($A_s = 1.0$ to $A_s = 11$).

Figure 2 illustrates typical elution patterns for SRM 870. Five of the columns utilized are known to contain embedded polar functional groups within the stationary phase to improve chromatographic performance toward bases. The separation of SRM 870 was similar for these columns. In each case, quinizarin eluted last, and both amitriptyline and quinizarin exhibited symmetrical peak shape (e.g., Figure 2A).

Peak asymmetry data for quinizarin and amitriptylin are plotted in Figure 6. The scatter in the data indicates independence of the two terms. Thus, it is possible for a column to exhibit high activity toward chelating agents and low activity toward bases, or other combinations (e.g., Figures 2C through 2F).

REFERENCES

- [1] Snyder, L.R.; Kirkland, J.J.; *Introduction to Modern Liquid Chromatography*; 2nd edition, New York, Wiley-Interscience (1979).
- [2] Sander, L.C.; Wise, S.A.; *SRM 869a Column Selectivity Test Mixture for Liquid Chromatography Polycyclic Aromatic Hydrocarbons*; Certificate of Analysis, NIST, Gaithersburg, MD (1998).
- [3] Phinney, K.W.; Sander, L.C.; *SRM 877 Chiral Selectivity Test Mixture for Liquid Chromatography*; Certificate of Analysis, NIST, Gaithersburg, MD (2000).
- [4] Neue, U.D.; Serowik, E.; Iraneta, P.; Alden, B.A.; Walter, T.H.; *Universal Procedure for the Assessment of the Reproducibility and the Classification of Silica-Based Reversed-Phase Packings I. Assessment of the Reproducibility of Reversed-Phase Packings*; J. Chromatogr. A, Vol. 849, pp. 87-100 (2000).
- [5] Engelhardt, H.; Arangio, M.; Lobert, T.; *A Chromatographic Test Procedure for Reversed-Phase HPLC Column Evaluation*; LC GC, Vol. 15, pp. 856-866 (1997).
- [6] Nacalai Tesque, Inc.; *Product Catalog*; Kyoto, Japan (1998).

Report Revision History: 19 December 2002 (Updated specifications in the <i>Chromatographic Conditions</i> section to expand the utility of the test and removed data collected under previous test conditions); 30 October 2000 (original report date).

Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Group at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet <http://www.nist.gov/srm>.

Table 1. Information Value Mass Fractions and Relative Areas for Components in SRM 870

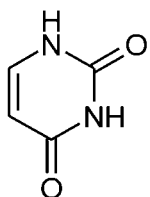
Component	CAS Number ^a	Property Evaluated	Source	Lot	Purity ^{b,c}	Mass Fraction ^b μg/g	Relative Area ^b 254 nm	Relative Area ^b 210 nm	Relative Area ^b 480 nm
Methanol	67-56-1		J. T. Baker	L30330	-				
Uracil	66-22-8	void volume marker	Aldrich	MS15011BS	98	28	0.02	0.00	
Toluene	108-88-3	hydrophobic retention, efficiency	Burdick and Jackson	AH700	-	1400	0.02	0.18	
Ethylbenzene	100-41-4	methylene selectivity, hydrophobic retention, efficiency	Aldrich	PS10785MS	99.8	1700	0.03	0.20	
Quinizarin	81-64-1	activity toward chelators	Aldrich	03116HS	97.9	94	0.10	0.01	1.00
Amitriptyline	549-18-8	activity toward bases	Sigma	48H0468	99.6	2800	0.83	0.61	

^aChemical Abstract Registry Number^bData are provided for information only as an aid in peak identification, and are not to be used for quantification purposes.^cPurity data provided by the manufacturer as % mass fraction.

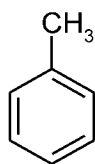
APPENDIX A. Participants

The following individuals and organizations participated in the development and evaluation of SRM 870:

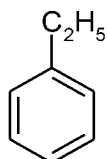
G. Boone, Varian, Inc., Harbor City, CA
J. DeStefano, Hewlett-Packard Co., Newport, DE
K. Harrison, The Separations Group, Inc., Hesperia, CA
R. Henry, Keystone Scientific, Inc., Bellefonte, PA
J. Higgins, Higgins Analytical, Mountain View, CA
B. Hornbake, Macherey-Nagel, Eastin, PA
J. Lamb, Hypersil, Astmoor, Runcorn, England
U. Neue, Waters, Milford, MA
M. Przybyciel, ES Industries, Marlton, NJ
M. Woelk, MetaChem Technologies, Inc., Torrance, CA
V. Yearick, Supelco, Inc., Bellefonte, PA
C. Young, R. Weigand, Alltech Associates, Inc., Deerfield, IL
K. Zimmerman, Mac-Mod Analytical, Inc., Chadds Ford, PA



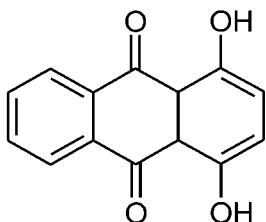
uracil - void volume marker



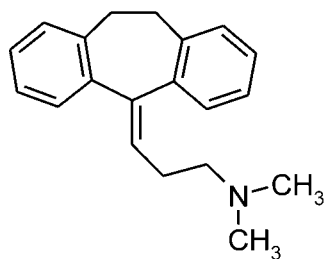
toluene - hydrophobic retention



ethylbenzene - methylene selectivity



quinizarin - activity towards chelating reagents



amitriptyline - activity towards bases

Figure 1. Structures and properties evaluated for components in SRM 870

Representative Separations of SRM 870

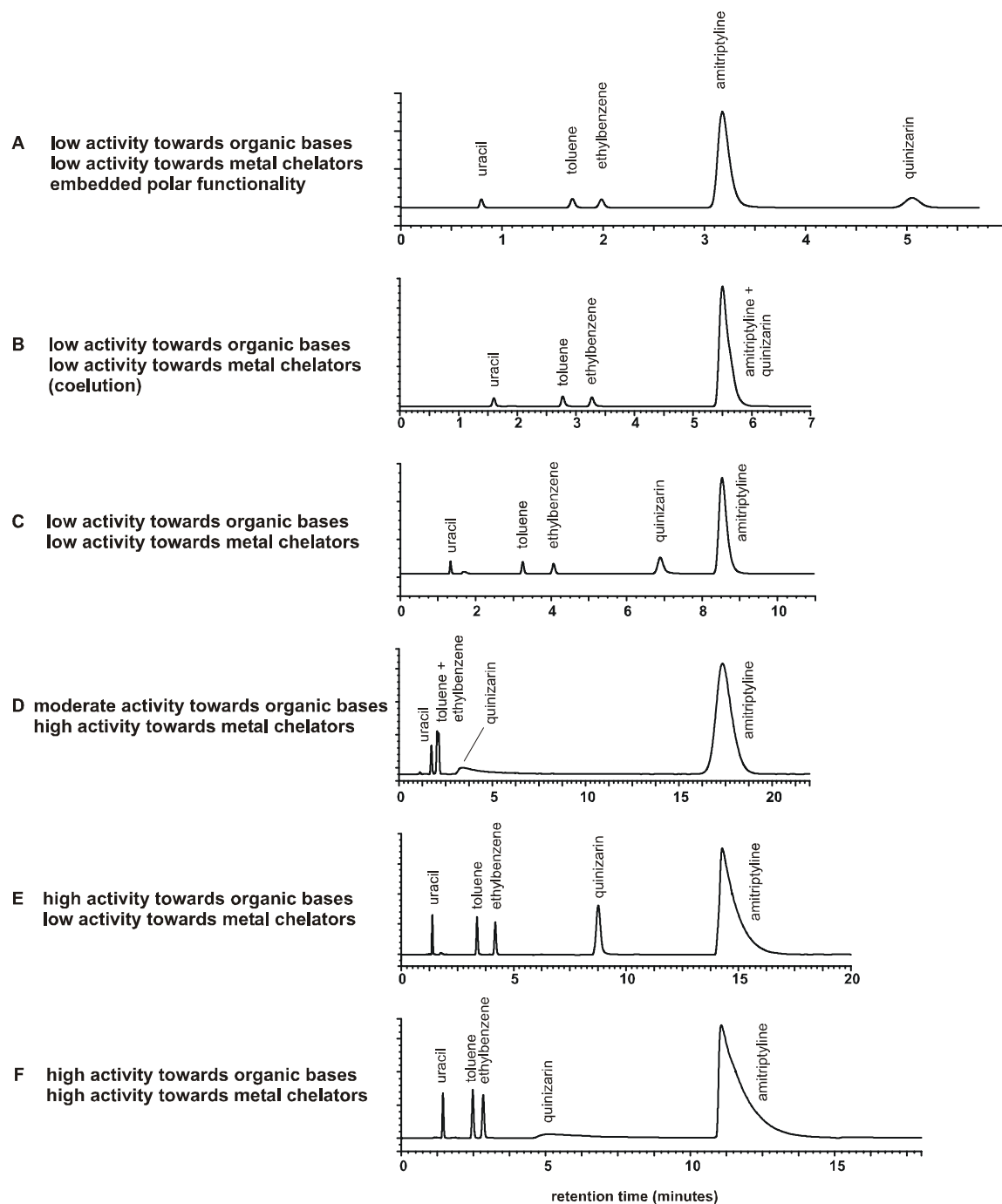


Figure 2. Examples of separations of SRM 870 on commercial C₁₈ columns

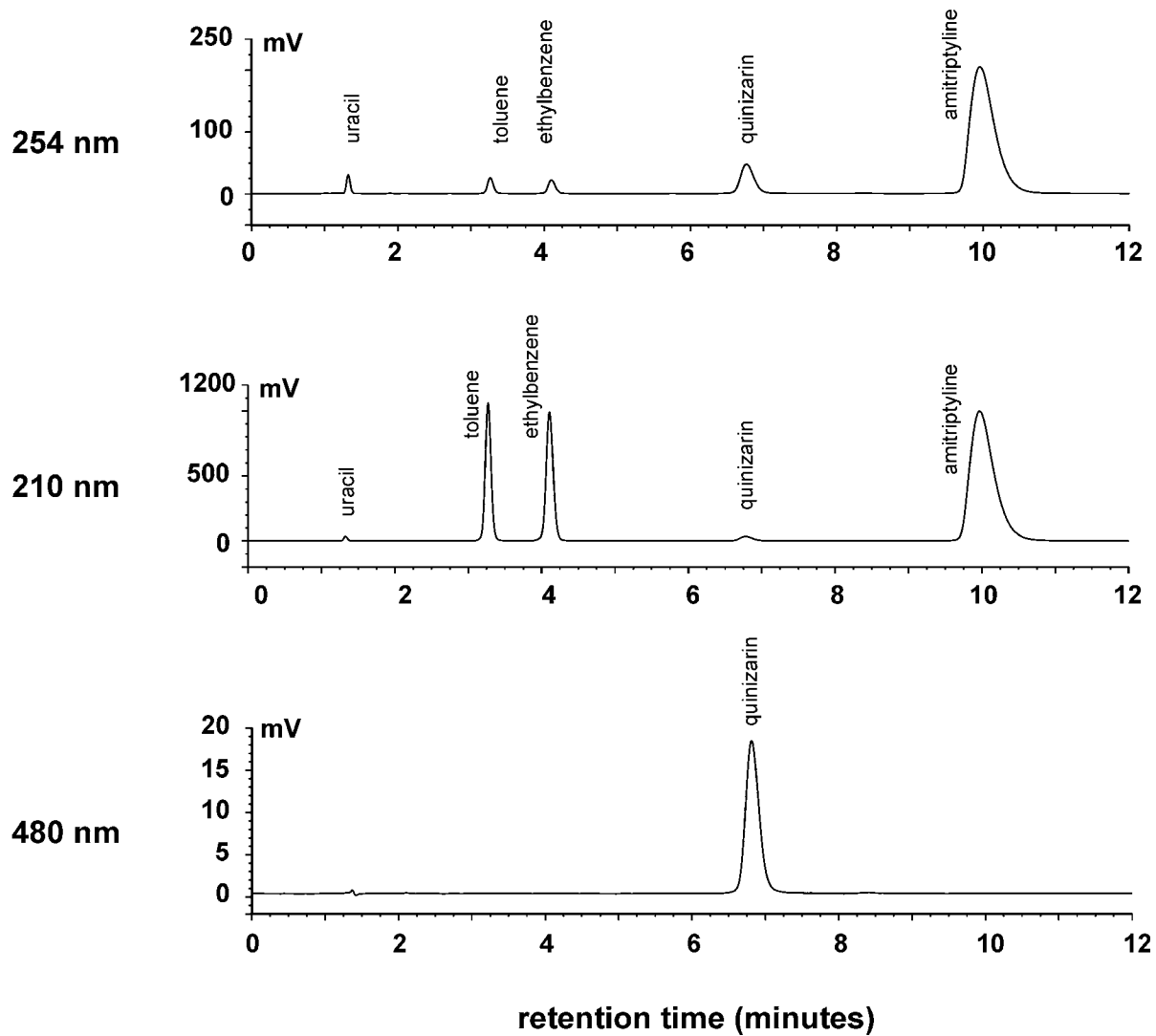


Figure 3. Separations of SRM 870 with detection at 254 nm, 210 nm, and 480 nm

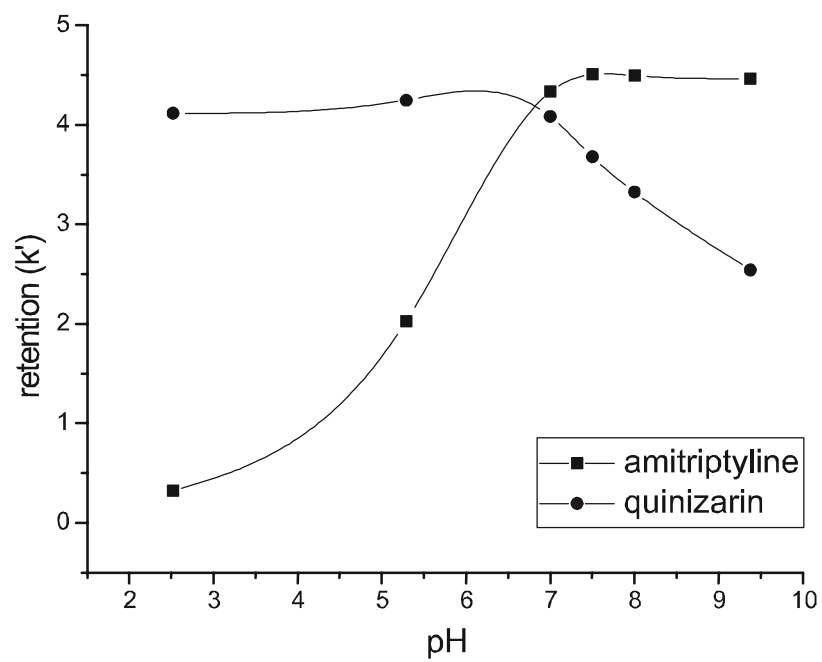


Figure 4. Plot of retention vs. pH for amitriptyline and quinizarin

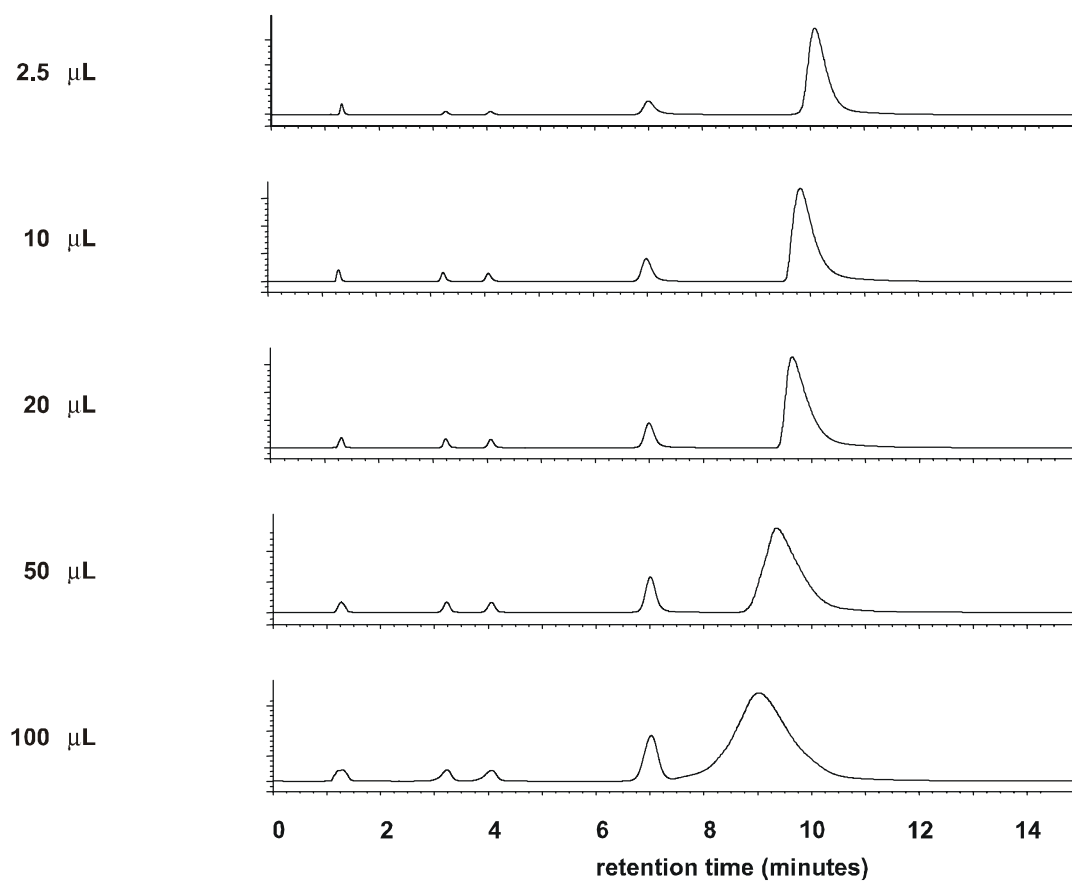


Figure 5. Separations of SRM 870 for different injection volumes

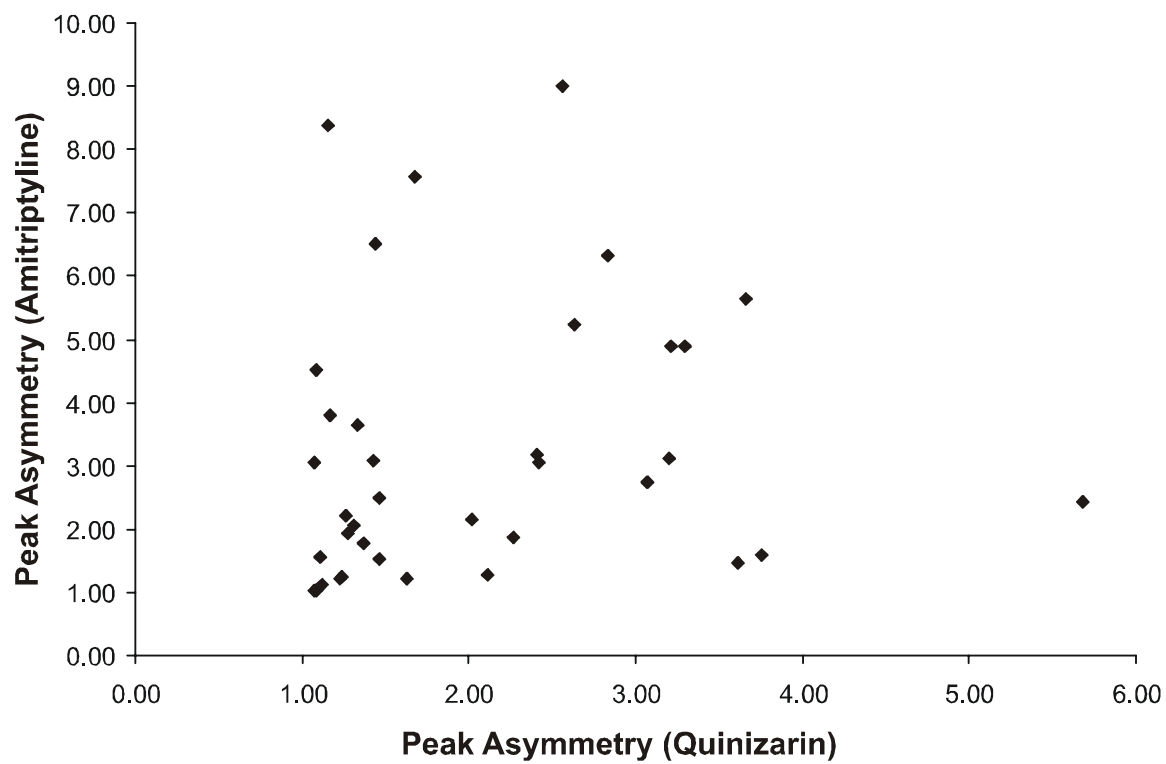


Figure 6. Plot of peak asymmetry for amitriptyline vs. peak asymmetry for quinizarin for various C₁₈ columns